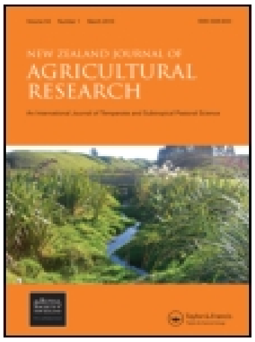

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Ryegrass endophyte, incidence, and control

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Abstract There was a high incidence of a fungus endophyte in most of the perennial ryegrass pastures and seed lines examined. The fungus was still viable in seed which had been stored at 0–5°C for 7 years. The main means of endophyte dissemination appear to be the sowing of infected seed and the re-seeding of infected plants in pastures. Field observations showed that when endophyte-free seed was sown, a set of mown plots and a grazed pasture were still free of endophyte 4 years after establishment. Endophyte-free seedlings were obtained by treating infected seeds with the fungicides propiconazole or prochloraz at 0.5 g/kg of seed. The fungus was eradicated from infected plants growing in pots by drenching the soil with a suspension of benomyl at 0.1 g per litre of growing medium.

Keywords Ryegrass staggers; perennial ryegrass; *Lolium perenne* L.; fungicides; benomyl; propiconazole; prochloraz; *Lolium* endophyte

INTRODUCTION

Ryegrass staggers, a disorder of grazing animals, has recently been associated with the presence of an endophytic fungus in ryegrass (Fletcher & Harvey 1981; Harvey, pers. comm.). The fungus has an *endoconidium* microspore stage in culture but no perfect stage has been recorded (Neill 1941). Endophyte mycelium occurs intercellularly in all above-ground parts of infected perennial ryegrass (*Lolium perenne* L.). Infected seed germinates to produce seedlings infected with mycelium and this is the only known way in which the fungus is spread (Neill 1940).

Neill (1940) and Lloyd (1959) found that many seed lines of New Zealand perennial ryegrass cultivars contain a high incidence of endophyte mycelium. This paper describes a survey of the current incidence of endophyte in ryegrass pastures, seed lines, and cultivar parent plants. Methods for eradicating endophyte from infected seeds and plants were examined, and control of the fungus is discussed in the light of results obtained.

EXPERIMENTAL DETAILS AND RESULTS

Detection of endophyte in plants and seeds

A leaf together with its sheath was removed from each plant to be examined and a strip of the upper epidermis was pulled from the leaf sheath with forceps. When mounted in 0.1% lactophenol cotton blue on a slide, endophyte mycelium in the epidermis appeared as septate, sparsely branched hypha generally running longitudinally between cells.

Endophyte in seeds was detected indirectly by germinating seeds in sterilised soil, and examining seedling leaf sheaths 3–5 weeks later for the presence of the fungus.

Incidence of endophyte in ryegrass plots and pastures

At DSIR Palmerston North, 4-year-old mown plots each sown with 1 of 10 different ryegrass cultivars were sampled for endophyte by taking tillers from 50 randomly selected plants in each plot. The cultivars and the incidence of endophyte infection are given in Table 1.

Fifty plants from each of 9 perennial ryegrass (*Lolium perenne* L.) pastures which varied in age from 2 to 15 years were also sampled for endophyte. The incidence varied from 0 to 96% of the plants per pasture infected, the average being 66%. The one pasture free from endophyte was a 4-year-old experimental pasture of cv. 'Ruanui'.

Incidence of endophyte in ryegrass seeds

Twenty-nine freshly harvested seed lines of perennial ryegrass, Italian ryegrass (*L. multiflorum* Lam.), and hybrid ryegrass (*L. perenne* × *L. multiflorum*) were sown in 10 cm pots of sterilised soil, and 20 seedlings from each line were examined 21 days later for the presence of endophyte mycelium.

Table 2 lists the incidence of viable endophyte present in these seed lines. The incidence of endophyte varied greatly among perennial ryegrasses. Only 1 of the 20 seed lines was free of the fungus and the average level of infection in each line was 51%. One of the 6 hybrid ryegrass lines contained a trace of endophyte and the 3 Italian ryegrass lines were free of the fungus.

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Table 1 Percentage of plants with endophyte in 4-year-old mown plots of ryegrass cultivars, 50 plants examined per plot.

Species	Cultivar	Endophyte infection (%)
Lolium perenne	Aberystwyth S 23	0
	Grasslands Nui	70
	Grasslands Ruanui	0
	RvP Vigor	0
	Yates Ellett	68
L. perenne × *L. multiflorum*	Aberystwyth Augusta	0
	Aberystwyth Sabrina	0
	Grasslands Ariki	10
	Grasslands Manawa	0
L. multiflorum	Grasslands Moata	0

Table 2 Incidence of endophyte in 20 seedlings grown from seed of each of 29 freshly harvested lines of ryegrass seed.

Ryegrass type	Cultivar	Number of seed lines tested	Endophyte infection (%) Range	Average
Perennial	Ellett	5	75–95	85
	Nui	8	15–65	33
	Ruanui	2	0–10	5
	Uncertified	5	45–80	64
Hybrid	Ariki	2	0	0
	Manawa	4	0–5	1
Italian	Paroa	3	0	0

Viability of endophyte in seed stored at low temperature

Eight seed lines of 'Nui' and 'Ellett' perennial ryegrass harvested over the period 1975–80 were stored at 5°C and a relative humidity of 50% until November 1980 and thereafter at 0°C and 25% relative humidity. Seed harvested in January 1981 was stored under these latter conditions. In February 1982, seeds of these lines were sown in pots of soil and 4 weeks later, 20 seedlings from each line were examined for endophyte. The results are given in Table 3. It is obvious that the endophyte remained viable in seed for at least 7 years when stored under these conditions.

Table 3 Incidence of endophyte in seedlings grown from seed stored for different periods at 0–5°C and 25–50% relative humidity.

Cultivar	Periods of storage (years)	Endophyte infection (%) (20 seedlings/line)
Nui	7	40
Nui	6	40
Ellett	6	80
Nui	5	5
Nui	3	70
Ellett	3	90
Nui	2	65
Nui	1	15

Control of endophyte

Hot water treatment of seed

A line of 9-month-old perennial ryegrass seed with about 95% endophyte infection was used in trials. Small muslin bags, each containing about 200 seeds, were soaked in water at 23°C for 4 h. Six bags were then placed in each of 3 water baths running at 53°, 55°, and 57° ± 0.1°C, respectively. After 5, 10, 15, 20, 25, and 30 min, a bag was removed from each bath and cooled in running tap water. Two additional bags were treated in a 59°C water bath for 10 and 20 min respectively and, as a control, another bag received no treatment other than the preliminary soaking. After treatment, 100 seeds from each bag were placed on moistened filter paper in petri dishes and 50 seeds were sown in a 10 cm pot of sterile soil. Germination counts were made in the petri dishes after 7 and 14 days at 25°C, and 10 seedlings from each pot of soil were examined for endophyte 28 days after sowing.

The effect of heat treatment on seed germination and seedling shoot length in petri dishes and the incidence of endophyte in seedlings growing in soil is shown in Table 4. Endophyte was eradicated from

Table 4 Germination, seedling shoot length, and endophyte infection in seedlings after different hot water seed treatments.

Treatment		At 14 days		Endophyte
Water temp. (°C)	Time (min)	germination (%)	Shoot length (mm)	infection (%)
53	5	94	72	90
	10	86	67	100
	15	84	83	80
	20	93	62	80
	25	87	61	90
	30	72	70	40
55	5	88	60	80
	10	81	83	50
	15	86	75	50
	20	73	52	40
	25	66	54	10
	30	51	58	10
57	5	74	73	70
	10	61	48	10
	15	47	49	0
	20	25	46	0
	25	16	47	0
	30	2	46	0
59	10	12	33	0
	20	0	0	–
Control		88	91	90

Table 5 Incidence of endophyte in seedlings grown from fungicide-treated seed.

Fungicide			Effectiveness of endophyte control
Common name	Formulation		
Benodanil	Calirus	50WP	Nil
Captan	Captan	80WP	
Carboxin	Vitavax	75WP	
Etridiazole	Terrazole	35WP	
Furmecyclox	Campogran	40WP	
Iprodione	Rovral	50WP	
Metalaxyl	Ridomil	25WP	
Ofurace	Chevron 20615	50WP	
Phosetyl	Aliette	80WP	
Pyroxyfur	Dowco 444	72EC	
Benomyl	Benlate	50WP	Incomplete
Dichlorobutrazol	Vigil	12.5EC	
Triadimefon	Bayleton	25WP	
Triadimenol + fuberidazole	Baytan F 17	15+2WP	
Etaconazole	Sonax	10WP	Complete
Prochloraz	Sportak	40EC	
Propiconazole	Tilt	25EC	

seed only after it had been treated for 15–30 min at 57°C or 10 min at 59°C. However, these treatments adversely affected both seed germination and seedling size.

Fungicide treatment of seed

A line of perennial ryegrass with about 95% endophyte infection was divided into 100-seed samples. One sample was shaken with an excess of each fungicide (Table 5) and sown in a 10 cm pot of sterilised soil. Fungicides available as liquid formulations were applied at 6 g a.i./kg of seed. Where phytotoxicity prevented seedling emergence, the fungicide was retested at a lower concentration. Ten seedlings from each treatment were examined for the presence of endophyte mycelium 25–40 days after sowing.

Table 6 Numbers, height, and endophyte infection of seedlings after different fungicide seed treatments.

Seed treatment Fungicide	g.a.i./ kg seed	Mean number of seedlings (20 sown/pot) 7 days	14 days	Mean seedling height (mm at 21 days)	Percent of seedlings infected with endophyte
			Trial 1		
Nil	-	18.1	19.4	114.2	95
Propiconazole	1.0	3.6**	15.8	42.4**	0
	0.5	7.2**	17.3	54.6**	0
	0.25	11.2**	19.2	76.1**	25
	0.1	13.9**	18.7	93.3**	85
Etaconazole	1.0	9.4**	18.0	41.7**	0
	0.5	10.2**	17.3	77.2**	15
	0.25	10.7**	18.7	90.9**	25
	0.1	14.0**	19.0	107.6	85
			Trial 2		
Nil	-	19.0	19.4	136.6	100
Propiconazole	0.5	8.6**	15.5**	68.9**	0
Prochloraz	1.0	9.8**	16.6**	73.9**	0
	0.5	12.0**	17.7**	90.0**	0
	0.25	15.8**	18.5*	123.6	15
	0.1	17.3*	18.3*	139.6	55

Table 5 lists the effectiveness of the fungicides in controlling endophyte. Three fungicides, etaconazole, propiconazole, and prochloraz showed promise but adversely affected seedling growth. Further trials with these fungicides were undertaken to determine the optimum concentration for control of endophyte with minimum phytotoxicity.

Plastic pots of 220 ml capacity with no drainage holes were filled with 220 g of non-sterile field soil. Twenty seeds were sown in each pot and 10 pots were sown for each treatment. Pots were placed in a water bath held at 15°±0.5°C and made up to a constant weight with water every 2 days during the trial. Two trials were run using different rates of the 3 fungicides. Untreated seeds were included as controls. Numbers of emerged seedlings in all pots were counted at 7 and 14 days, height of seedlings in 1 pot was measured at 21 days, and endophyte infection was determined 35 days after sowing. Rates of fungicide application and results are given in Table 6.

Propiconazole, etaconazole, and prochloraz were phytotoxic at rates which eradicated endophyte from seed. Seedling emergence at 7 days was reduced by all 3 fungicides and the number of seedlings emerged after 14 days was often less than for non-treated controls. In the second trial no further seedlings emerged from 14 to 21 days.

Seedling height 21 days after sowing was significantly reduced ($P<0.01$) by the 3 fungicides at rates which killed endophyte. The first 1 or 2 leaves were distorted, curled, and very dark green, some seedlings being more affected than others. Seed treated with prochloraz produced plants which were less affected than those from propiconazole or etaconazole-treated seed. Subsequent leaves on all plants appeared to be normal.

Both prochloraz and propiconazole completely eradicated endophyte from seeds when used at 0.5 g/kg of seed but control was incomplete at half this amount. Etaconazole did not kill endophyte in all seeds treated with 0.5 g/kg of seed.

Eradication of endophyte mycelium in ryegrass plants

Benomyl, although only partially effective as a seed dressing for killing endophyte (Table 5), was the only non-phytotoxic fungicide capable of killing endophyte. It was tested for its effect on endophyte mycelium in plants.

Five perennial ryegrass plants infected with endophyte were dug from pastures, split into ramets, and planted in a sand/peat mixture in undrained 220 ml plastic pots. Three pots of each of the 5 ryegrass clones were dosed with benomyl solutions at concentrations of 0.025 g, 0.05 g, or 0.1 g of benomyl per litre of sand/peat 4 weeks later. Control pots had only water added. Pots were made up to weight with a nutrient solution every 2–3 days during the trial and plants were examined for endophyte mycelium after 8 weeks.

Benomyl at 0.1 g/litre killed the mycelium in all 15 treated plants, at 0.05 g/litre 8 of the 15 plants were free of endophyte, and at 0.025 g/litre only 1 of the 15 plants was free. No phytotoxicity was apparent. Endophyte was present in all control plants.

Parent plants of 4 Grasslands Division, DSIR ryegrass cultivars and 3 experimental lines were examined for endophyte. All parent plants of

'Ruanui' and 'Manawa' were free of endophyte but only 2 of the 22 'Nui' and 2 of the 8 'Ariki' parent plants were free. All parent plants of the 2 perennial ryegrass experimental lines contained endophyte mycelium and the Italian ryegrass experimental line was entirely free of the fungus.

Ramets from all parent plants of 'Nui' and 'Ariki' were treated with benomyl at 0.2 g and 0.1 g/litre using the method described. Both rates of benomyl were effective in eliminating endophyte mycelium.

DISCUSSION

Most of the perennial ryegrass pastures and seed lines tested for endophyte were heavily infected. This finding is in agreement with earlier surveys by Neill (1940) and Lloyd (1959). They too, noted that seed harvested from long established pastures had a particularly high incidence of the fungus. However, our survey showed also that seed harvested from newly sown pastures of 'Nui' and 'Ellett' had a high incidence of endophyte. These 2 cultivars were derived from a natural ecotype ryegrass growing at Mangere near Auckland and they, like many old permanent pastures, exhibit good persistence when growing in adverse conditions. The dominance of endophyte-infected plants in these old pastures suggests that the endophyte is not harmful to ryegrass.

The mycelium of the endophyte fungus is present in all seed from infected plants. Neill (1940) has shown that this mycelium dies if seed is stored for more than 18 months and he suggested that infected seed be sorted for this period so that an endophyte-free pasture could be obtained. This is not practical on a large scale. Moreover, we have shown that the endophyte can survive for at least 7 years under some storage conditions.

The present variability in the incidence of endophyte in certified seed lines is the result of the mechanics of the seed certification process. At Grasslands Division, a nucleus isolation stock of seed is harvested from the parent plants of a cultivar every 4–5 years. Some of this seed is immediately sown for multiplication; the rest is stored under varying conditions and sown for further multiplication during the next few years. The precise storage condition and age of seed will influence viability of endophyte in the nucleus isolation seed and ultimately the incidence of endophyte in lines of seed derived from it.

The majority of 'Nui' and 'Ariki' parent plants contained endophyte mycelium whereas the parent plants of 'Ruanui' were free of the fungus. 'Ruanui' is the original New Zealand certified perennial ryegrass, the seed of which was found by Neill (1940) and Lloyd (1959) to contain endophyte. The reason for it being now free of endophyte is that in 1976 a fresh isolation was made from progeny of the original parents. These plants had been grown in 1972 from nucleus seed harvested in 1962. This seed was low in germination and presumably the endophyte was dead. All 'Ruanui' plants from this fresh isolation were free of endophyte. We examined only 2 lines of 'Ruanui' seed in our survey. The low incidence of endophyte in one line could be caused by contamination from volunteer plants in the pasture or by infection of plants with endophyte. The parent plants of 'Manawa' were free of endophyte and so too were most seed lines of 'Manawa' and 'Paroa'. Neill (1940) and Lloyd (1959) found that Italian ryegrass and hybrids between Italian and perennial ryegrasses were free of endophyte. We were unable to find endophyte in Italian ryegrass but detected a trace in one line of 'Manawa' seed. The parents of 'Manawa' were free of endophyte mycelium and so this trace infection may have been from contaminating seed. 'Ariki' is also a hybrid ryegrass but the majority of its parent plants are infected with endophyte. This cultivar is a backcross between 'Ruanui' and 'Manawa' and its susceptibility to endophyte may result from the dominance of perennial ryegrass parentage.

The simplest and surest way to have endophyte-free seed is to harvest it from endophyte-free plants. Parent plants of commercial cultivars can be freed from endophyte by drenching benomyl into the soil around plants growing in pots. Endophyte-free seed can then be bulked up from these cultivar parent plants. Four to five years will elapse before seed from endophyte-free parents of 'Nui' and 'Ariki' becomes generally available to the farming community because it will take this time to bulk the seed. In the meantime endophyte-infected seed can be treated with propiconazole or prochloraz at 0.5 g/kg of seed. Laboratory and field trials indicate that both fungicides are equally effective in killing endophyte, and seedling growth is checked only slightly in the first 2–3 weeks after sowing.

It is of little use establishing endophyte-free pastures if plants become infected within a few years. Neill (1940) was unable to infect ryegrasses with endophyte mycelium and we also have been unsuccessful. If plants do become infected in the field, evidence suggests that the rate is not rapid. We have found several mown swards and one pasture of perennial ryegrass which are free of endophyte 4 years after establishment. Pastures initially become infected through the sowing of infected seed. The incidence of plants with endophyte in pastures may increase through reseeding either from infected plants or from hay transferred from infected pastures. The possibility that endophyte mycelium or spores may be able to infect established plants is being further investigated.

Most perennial ryegrass pastures in New Zealand are likely to contain endophyte. Ryegrass staggers is widespread throughout the country and its incidence and severity have been increasing in recent years. Concurrent with this increase in ryegrass staggers has been the release of 3 new cultivars of ryegrass, all with high endophyte infection. If the association of endophyte and ryegrass plant should prove to be responsible for ryegrass staggers then the disorder could be controlled by establishing endophyte-free pastures. It would be an expensive and long-term operation to resow all ploughable land with endophyte-free seed. A search must be continued for systemic fungicides which will eradicate endophyte mycelium from ryegrass pastures.

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